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(54) Thermostable luciferase of a firefly, gene of a thermostable luciferase of a firefly, novel recombinant DNA, and process for the preparation of a thermostable luciferase of a firefly.

(57) The present invention relates to a thermostable luciferase of a firefly wherein an amino acid at the 217-position of the amino acid sequence of the wild-type firefly luciferase or an amino acid equivalent to the amino acid at the 217-position of the luciferase of GENJI firefly or HEIKE firefly is converted into a hydrophobic amino acid, a gene encoding said thermostable luciferase, a vector comprising the gene encoding said thermostable luciferase inserted therein, and a process for the preparation of a thermostable firefly luciferase comprising the use of said vector.

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The present invention relates to a thermostable luciferase of a firefly, a gene of a thermostable luciferase of a firefly, novel recombinant DNA, and a process for the preparation of a thermostable luciferase of a firefly.

Luciferase is a luminescence enzyme which catalyzes oxidation of luciferin, thus allowing it to emit light. Luciferase derived from fireflies, such as GENJI firefly, HEIKE firefly, American firefly requires ATP, etc., for the luminescence of luciferin, and owing to this property, luciferase is used for the quantitative determination of a trace amount of the above-mentioned ATP, etc.

However, since luciferase is generally unstable to heat, this enzyme has the defect of being liable to be inactivated in storage as a reagent. As a means of avoiding this disadvantage, the addition of a salt, etc., makes it possible to store luciferase stably to some extent. In this case, however, the presence of the salt sometimes leads to another disadvantage, i.e. hindrance of luciferase reaction.

As a result of extensive research the present inventors have found that the above object can be solved by converting an amino acid residue at a specific position into a hydrophobic amino acid residue.

That is, the present invention comprises:

1. A Gene of a thermostable luciferase of a firefly, which encodes the amino acid sequence of a wild-type firefly luciferase in which an amino acid at the 217-position or an amino acid at the position equivalent to the 217-position of the luciferase of Luciola cruciata (GENJI firefly) or Luciola lateralis (HEIKE firefly) is replaced by a hydrophobic amino acid.

2 Gene of a thermostable luciferase of a firefly according to claim 1, wherein the wild-type firefly luciferase is the luciferase of Luciola lateralis (HEIKE firefly) or Luciola cruciata (GENJI firefly).

3. Gene of a thermostable luciferase of a firefly according to claim 1 or 2, wherein the hydrophobic amino acid is isoleucine, leucine, or valine.

4. Recombinant DNA, which comprises the gene of a thermostable luciferase of a firefly according to any one of claims 1 to 3 inserted into a vector DNA.

5. Process for the preparation of a thermostable luciferase of a firefly, which comprises incubation in a culture medium of a microorganism belonging to the genus Escherichia, carrying the recombinant DNA according to claim 4 and being capable of producing a thermostable luciferase of a firefly, and subsequent collection of the thermostable luciferase of the firefly from said culture medium.

6. Thermostable luciferase of a firefly, wherein an amino acid at the 217-position of the amino acid sequence of a wild-type firefly luciferase or an amino acid at the position equivalent to the 217-position of the luciferase of Luciola cruciata (GENJI firefly) or Luciola lateralis (HEIKE firefly) is replaced by a hydrophobic amino acid.

7. Thermostable luciferase of a firefly according to claim 6, wherein the wild-type firefly luciferase is the luciferase of Luciola lateralis (HEIKE firefly) or Luciola cruciata (GENJI firefly).

Figure 1 shows a cleavage map of the recombinant plasmid DNA pALf indicating the recognition sites of various restriction enzymes.

Figure 2 shows a cleavage map of the recombinant plasmid DNA pGLf indicating the recognition sites of various restriction enzymes.

The main object of the invention is to develop a thermostable luciferase of a firefly.

The invention is described in detail hereinafter.

As a precondition for providing a thermostable luciferase by the present method of gene mutation, it is necessary to prepare the gene of a wild-type firefly and its recombinant DNA.

The types of wild-type firefly genes, are adopted depending on the type of thermostable luciferase gene attempted. Any types of genes can be used if they are derived from fireflies such as Luciola cruciata (GENJI firefly) or Luciola lateralis (HEIKE firefly). These genes are prepared according to the method known in the art. For example, a wild-type firefly gene and its recombinant DNA can be prepared according to the method as disclosed in Japanese Patent Appln. LOP Publication No. 51,086/1989.

In the present invention, "an amino acid at the position equivalent to the 217-position of luciferase of GENJI firefly or HEIKE firefly" means an amino acid which corresponds to the amino acid at the 217-position of the amino acid sequence of luciferase of GENJI firefly or HEIKE firefly, as a result of the comparison of the established luciferase amino acid sequence with the amino acid sequence of luciferase of GENJI firefly or HEIKE firefly.

Specifically, this is determined by comparison of the homology between the amino acid sequence of each luciferase and the amino acid sequence of GENJI or HEIKE firefly using commercially available software for analysis of homology, e.g. Micro Genie™ (manufactured by Beckman). Although the corresponding amino acids are not particularly limited, they are e.g. threonine or alanine.

In the present invention, "a hydrophobic amino acid" includes isoleucine, leucine, valine, methionine, tryptophan, phenylalanine, proline, cysteine, and alanine. Among these amino acids, isoleucine, leucine, or

valine are particularly preferred due to their high hydrophobicity. Mutagenesis of the gene of a wild-type firefly luciferase can be effected according to the method known in the art, depending on attempted mutagenesis.

That is, it is possible to adopt a broad scope of methods: e.g. a method comprising allowing a chemical as mutagen to contact and act on the wild-type firefly luciferase gene or the recombinant DNA having said gene inserted; an ultraviolet irradiation method; a means of genetic engineering; and a means of protein engineering

Chemicals acting as mutagen for use in said mutagenesis include e.g. hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (NTG), nitrous acid, sulfurous acid, hydrazine, formic acid, 5-bromouracil.

Conditions for the application can vary depending on the type of chemicals used, and these conditions are not particularly limited, insofar as the mutagenesis of the wild-type firefly luciferase gene is virtually brought about as desired.

Mutagenesis by ultraviolet irradiation can also be effected according to the conventional method as described above (Gendai Kagaku (Japan), 24-30, the June issue of 1989).

As a method of making full use of protein engineering, a means generally known as site-specific mutagenesis can be employed: e.g. the Kramer method (Kramer, W. et al., Nucleic Acids Res, 12: 9,441-9,456 (1984) : Kramer, W. et al., Methods Enzymol, 154: 350-367 (1987): Bauer, C. E. et al., Gene, 37: 73-81 (1985)), the Eckstein method (Taylor, J. W. et al., Nucleic Acids Res, 13: 8,749-8,764 (1985): Taylor, J. W. et al., Nucleic Acids Res, 13: 8,765-8,785 (1985): Nakamaye, K. L. et al., Nucleic Acids Res, 14: 9,679-9,698 (1986)), the Kunkel method (Kunkel. T. A., Proc. Natl. Acid Sci. U.S.A., 88: 488-492 (1985): Kunkel. T. A. et al., Methods Enzymol, 154: 367-382 (1987)).

In addition to the above-mentioned gene mutagenesis method, it is of course possible to directly synthesize a desired modified luciferase gene of firefly through organic or enzymatic synthesis.

The determination and identification of the base sequence of a desired luciferase gene of firefly can be effected e.g. through the chemical modification method as described by Maxam-Gilbert (Maxam-Gilbert, Meth. Enzym., 65: 499-560 (1980)), the dideoxynucleotide termination procedure using M13 phage (Messing et al., Gene, 19: 269-276 (1982)).

The gene of a thermostable luciferase prepared as described above can be inserted into vectors, such bacteriophages, cosmids, or plasmids used for the transformation of procaryotic or eucaryotic cells, according to the conventional method. Subsequently, the resulting vectors can be used for the transformation or transduction of hosts at which said vector to be directed, in accordance with the known method in the art.

In case bacteria belonging to the genus Escherichia, e.g E. coli JM101 (ATCC 33876), E. coli DH1 (ATCC 33849), E. coli HB101 (ATCC 33694) are selected as hosts, they can be transformed by the method as described by Hana-han (DNA Cloning, 1: 109-135 (1985) or transduced by the method as described in Maniatis et al. (Molecular Cloning, 256-268, Cold Spring Harbor laboratory (1982)), whereby their transformed or transduced strain can be obtained.

A bacterial strain belonging to the genus Escherichia, carrying a recombinant DNA having the gene of a thermostable firefly luciferase inserted into the vector DNA and being capable of producing a thermostable firefly luciferase can be obtained from the above bacterial strain by screening the bacterial strain with the ability to produce a thermostable firefly luciferase.

Novel recombinant DNA purified from the thus obtained bacterial strain can be prepared according to the method described e.g. by P. Guerry et al. (J. Bacteriology, 116: 1,064-1,066 (1973)), D. B. Clewell (J. Bacteriology, 110: 667-676 (1972)).

In order to prepare DNA containing the gene of a thermostable firefly luciferase, the recombinant plasmid DNA thus obtained is digested e.g. with restriction enzymes such as Eco RI and Pst I at 30 to 40 °C, preferably about 37 °C, and for 1 to 24 hours, preferably about 2 hours, followed by subjection to agarose gel electrophoresis in accordance with the method as described in Maniatis et al. (Molecular Cloning, p. 150, Cold Spring Harbor laboratory (1982)), whereby the DNA containing said luciferase gene can be obtained.

For production of a thermostable firefly luciferase, it is possible to use a usual solid medium, but it is preferable to use a liquid medium as far as circumstances permit, in order to culture the bacteria strain belonging to the genus Escherichia, carrying the recombinant DNA having the gene of a thermostable firefly luciferase inserted into the vector DNA and being capable of producing a thermostable luciferase of a firefly.

The bacterial strain is cultured in a medium in which e.g. one or more inorganic salts among sodium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulfate, magnesium chloride, ferric chloride, ferric sulfate, manganese sulfate are added to one or more nitrogen sources among yeast extract, trypton, peptone, meat extract, corn steep liquor and exudate of soybean or wheat

bran, and if necessary, sugars (or carbohydrates), vitamins, etc., are suitably added.

The initial pH value of the culture medium is suitably adjusted to 7-9.

Cultivation is carried out at 30 to 42 °C, preferably about 37 °C, and for 4 to 24 hours, preferably 6 to 8 hours, and preferably effected by submerged aeration culture, shake culture, or stationary culture.

After cultivation, mutant luciferase is recovered from the culture according to the methods known in the art:

For example, the present enzyme can be extracted from bacteria bodies by disrupting the cells using ultrasonification and crushing; by treatment with a lytic enzyme such as lysozyme; or by autolysis of bacteria bodies in the presence of toluene, etc., with or without shaking. The lysate is filtered or centrifuged for removal of cells and cell debris.

If it is necessary to remove nucleic acids, streptomycin sulfate, protamine sulfate, or manganese sulfate is added to the filtrate or the supernatant.

The mixture is then fractionated by addition of ammonium sulfate, alcohols, acetone, etc., thereby giving precipitates as crude enzyme.

For the preparation of a purified enzyme standard, crude enzyme thus obtained may be purified by a method or the suitable combination of methods which include gel filtration by Sephadex, Ultro-Gel, or Bio-Gel; an adsorption-elution method by ion exchanger; electrophoresis by polyacrylamide gel, etc. ; an adsorption-elution method by hydroxyapatite; precipitation by sucrose density-gradient centrifugation, etc. ; affinity chromatography; and fractionation by molecular sieve membrane, hollow fiber membrane, etc.

According to the method as illustrated above, the desired thermostable luciferase of a firefy can be obtained. The present thermostable luciferase of a firefly is characterized by the following properties:

(1) Suitable working temperature range:

The suitable temperature for the action ranges from 0 to 65 °C.

(2) Inactivation conditions of pH, temperature, etc.:

i) The enzyme is completely inactivated at pH values of 4.0 or lower or 12.0 or more, 4 hours after.

ii) The enzyme is completely inactivated by heating at a temperature of 65 °C at pH 7.8, 60 minutes after.

(3) Thermostability:

80 % or more of enzyme activity remains at a temperature of 50 °C 20 minutes after, and 65 % or more of enzyme activity remains by heating at a temperature of 50 °C, even 60 minutes after.

Except for the above properties, the present enzyme is identical to the luciferase of the wild-type GENJI firefly as disclosed in Japanese Patent Appln. LOP Publication No. 141,592/1989 and of the wild-type HEIKE firefly as disclosed in Japanese Patent Appln. LOP Publication No. 262,791/1989.

The following Examples further illustrate the invention.

Sections (1) to (10) below describe the construction of DNA containing the gene encoding a luciferase of the firefly Photinus pyralis. (Said DNA is used as a probe for screening DNA containing the gene encoding the luciferase of the firefly Luciola cruciata).

(1) Preparation of m-RNA

1 g of dried tails of the firefly Photinus pyralis (manufactured by Sigma Co., Ltd) was sufficiently disrupted and ground in a mortar using a pestle, followed by addition of 5ml of a dissolution buffer [20 mM Tris-HCl buffer (pH 7.4), 10 mM NaCl, 3mM magnesium acetate, 5 % (W/V) sucrose, 1.2 % (V/V) Triton X-100, and 10 mM vanadyl nucleoside complex (produced by New England Biolabs]. The sample was further disrupted and ground in the same way as above, whereby a solution containing the disrupted tails of Photinus pyralis was obtained.

5 ml of the solution thus obtained was introduced into a cup type blender (manufactured by Nippon Seiki Seisakusho Co.) and blended at 5,000 r.p.m. for 5 min. 12 ml of a guanidine isocyanate solution (6 M guanidine isocyanate, 37.5 mM sodium citrate (pH 7.0), 0.75 % (W/V) sodium N-lauroylsarcosinate, and 0.15 M β-mercaptoethanol) was added to the sample solution. This solution was further blended at 3,000 r.p.m. for 10 min. using the above blender. The mixture was then filtered through a three-folded gauze, whereby a filtrate was obtained. Subsequently, the filtrate was pipetted and layered onto 4 ultracentrifugation tubes (manufactured by Hitachi Koki Co.) so as to be stacked on 1.2 ml of 5.7 M cesium chloride previously placed in each tube. The sample was centrifuged at 30,000 r.p.m. for 16 hours at a temperature of 15 °C (SCP55H ultracentrifuge manufactured by Hitachi Koki Co.), so that precipitates were obtained. The precipitates thus obtained were washed with cold 70 % (V/V) ethanol and were then suspended in 4 ml of 10 mM Tris-HCl buffer [10 mM Tris-HCl buffer (pH 7.4), 5 mM EDTA, and 1 % sodium dodecyl sulfate].

The mixture was extracted with an equal volume of n-butanol and chloroform (4 : 1 [V/V]). The extract was centrifuged over 10 min. at 3,000 r.p.m. in accordance with a conventional manner, whereby the solution was separated into an aqueous layer and organic solvent layer, and the former was saved. 4 ml of 10 mM Tris-HCl buffer of the same composition as above was added to the organic solvent phase. The organic phase was back-extracted twice in the same manner as described above, and the aqueous phase was pooled each time. To the combined aqueous phase, 1/10 volume of 3 M sodium acetate (pH 5.2) and two volumes of cold ethanol were added. The mixture was allowed to stand for 2 hours at a temperature of -20 °C and was then centrifuged at 8,000 r.p.m. for 20 min. in accordance with the conventional manner, whereby RNA was precipitated. The RNA thus obtained was dissolved in 4 ml water and was then precipitated with ethanol in the same manner as above.

The RNA obtained was resuspended in 1 ml water, and 3.75 mg RNA were obtained.

7 mg of RNA in total were prepared by repeating the above procedure. In order to separate m-RNA from RNA, 7 mg of the RNA were applied to oligo(dT)-cellulose (produced by New England Biolabs) column chromatography.

For column chromatography, the column was prepared as follows: 0.5 g of the resin was swelled with an eluting buffer [10 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, and 0.1 % (W/V) sodium dodecyl sulfate] and was then packed into a 2.5 ml Terumo syringe (manufactured by Terumo) used as a column, and the column was equilibrated with a binding buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.4 M NaCl, and 0.1 % sodium dodecyl sulfate].

An equal volume of a buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.8 M NaCl, and 0.1 % sodium dodecyl sulfate] was added to the RNA (7 mg) suspension. This mixture was heated at 65 °C for 10 min. and then rapidly cooled on ice. The sample thus treated was applied to an oligo(dT)-cellulose column as prepared above, and the resin was washed with the binding buffer, whereby unbound r-RNA and t-RNA were completely washed out. Then, m-RNA was eluted with the eluting buffer, so that 40 µg of m-RNA were obtained.

(2) Concentration of Luciferase m-RNA

Subsequently, luciferase m-RNA was concentrated by sucrose density-gradient centrifugation.

A 10 to 25 % (W/V) sucrose density-gradient was prepared as follows: 0.5 ml of a 40 % (W/V) sucrose solution [50 mM Tris-HCl buffer (pH 7.5), 20 mM NaCl, 1mM EDTA, and 40 % (W/V) sucrose] was introduced into a polyaroma tube for Rotor SW41 manufactured by Beckman, and on said sucrose solution was layered 2.4 ml of each sucrose (25 % (W/V), 20 % (W/V), 15 % (W/V), and 10 % (W/V)), and they were allowed to stand over 24 hours. Then, 30 µg of m-RNA were layered on the sucrose gradient, which in turn was centrifuged at 30,000 r.p.m. over 18 hours at a temperature of 18 °C in accordance with the conventional manner. After centrifugation, the sample was fractionated into 0.5 ml per fraction, and the m-RNA was recovered by ethanol precipitation and was resuspended in 10 µl water.

Then, a protein encoded by m-RNA was examined for identification of the fraction containing a high level of luciferase m-RNA. 1 µl of the fractionated RNA, 9 µl of rabbit reticulocyte lysate (produced by Amersham) , and 1 µl of [^{35}S] methionine (produced by Amersham) were mixed, and the mixture was allowed to react at 30 °C for 30 min After 150 µl NET buffer [150 mM NaCl, 5 mM EDTA, 0.02 % (W/V) NaN_3, 20 mM Tris-HCl buffer (pH 7.4), and 0.05 %(W/V) Nonidet P-40 (BR detergent)] and 1 µl of anti-luciferase serum (which was prepared according to the method as described below) were added to said mixture, it was allowed to stand at 4 °C for 18 hours.

Subsequently, 10 mg of protein A Sepharose (produced by Pharmacia AG) were added thereto, and the sample solution was allowed to stand at a temperature of 20 °C for 30 min., followed by centrifugation for 1 min. at 12,000 r.p.m. in accordance with a conventional manner, whereby the resin was recovered.

The recovered resin was washed 3 times with 200 µl NET buffer, and then 40 µl of a sample buffer for SDS-PAGE [62.5 mM Tris-HCl buffer (pH 6.8), 10 % (V/V) glycerol, 2 % (W/V) sodium dodecyl sulfate, 5 % (V/V) mercaptoethanol, and 0. 02 % (W/V) Bromophenol Blue] was added to said resin, which was then boiled for 3 min. at 100 °C and centrifuged at 12,000 r.p.m. for 1 min. in accordance with the conventional manner. The supernatant obtained in this centrifugation was recovered, and the whole amount of the supernatant was subjected to gel electrophoresis on a 7.5 % (W/V) sodium dodecyl sulfate-polyacrylamide gel according to the method as described by Laemmli [Nature, p. 227, p. 680 (1970)].

Following gel electrophoresis, the gel was immersed in 10 % (V/V) acetic acid for 30 min. whereby proteins were immobilized, followed by immersion in water for 30 min. and then in 1 M sodium salicylate for 30 min., and the gel was dried. Fluorography of the dried gel was conducted by exposure to X-ray film RX (manufactured by Fuji Film Co.).

According to the above procedure, the presence of a band on the film indicated the presence of an elevated level of luciferase m-RNA in that fraction, so that the concentrated luciferase m-RNA fraction could be identified.

(3) Preparation of Anti-luciferase Serum

Rabbit anti-luciferase serum against purified luciferase was prepared according to the following method.

0.7 ml of luciferase solution (3.2 mg/ml) [luciferase (Sigma) was dissolved in 0.5 M glycylglycine solution (pH 7.8)] is mixed with an equal volume of Freund's complete adjuvant]. The mixture was administered as an antigen into a Japanese white rabbit at the pad with a body weight of 2 kg.

Two weeks thereafter, the same amount of the antigen-adjuvant mixture was intracutaneously administered into the rabbit at the back. One week thereafter, the same procedure was carried out and then sacrificed and bled. The blood thus obtained was left standing stand over 18 hours at a temperature of 4 °C and then centrifuged over 15 min. at 3,000 r.p.m. according to the conventional manner, so that an anti-luciferase serum was obtained as a supernatant.

(4) Synthesis of c-DNA

Synthesis of c-DNA was carried out using a kit produced by Amersham.

2 μg of the m-RNA obtained above were treated according to the method described in Mol. Cell Biol., 2, 161 (1982) and Gene, 25, 263 (1983) as recommended by the manufacturer's instructions, whereby 300 ng of double-stranded c-DNA were obtained.

150 ng of the c-DNA were dissolved in 7 μl of a TE buffer [10 mM Tris-HCl buffer (pH 7.5) and 1 mM EDTA]. 11 μl of a mixture solution [280 mM sodium cacodylate (pH 6.8), 60 mM Tris-HCl (pH 6.8), and 2 mM cobalt chloride], 3.8 μl of a tailing mixture solution [7.5 μl of 10 mM dithiothreitol, 1 μl of 10 ng/ml polyA, 2 μl of 5 mM dCYP, and 110 μl water], and 29 units of terminal transferase (produced by Boehringer Mannheim GmbH) were added to the sample solution. This solution was allowed to react at 30 °C for 10 min, and then 2.4 μl of 0.25 M EDTA and 2.4 μl of 10 % (W/V) sodium dodecyl sulfate were added to the mixture to stop the reaction.

Subsequently, this sample solution was treated with 25 μl of water-saturated phenol for removal of proteins. The aqueous layer was recovered, and 25 μl of 4 M ammonium acetate and 100 μl of cold ethanol were added to the aqueous portion. The mixture was allowed to stand at -70 °C for 15 min. and was then centrifuged at 12.000 r.p.m. for 10 min., whereby the c-DNA was recovered. The c-DNA was dissolved in 10 μl TE buffer to give a solution of c-DNA. According to the above procedure, 100 ng of deoxycytidine-tailed c-DNA were obtained.

(5) Preparation of the Recombinant Plasmid DNA pMCE10 Used as a Vector

According to the method as described by T. Masuda et al. (AgricuLtd. ural Biological Chemistry, 50: 271-279 (1986)), plasmid DNA pKN305 and plasmid DNA pMC1403-3 (disclosed in Japanese Patent Appln. LOP Publication No. 274, 683/1986) were respectively constructed using E. coli strain W3110 (ATCC 27325), plasmid DNA pBR325 (produced by BRL), and plasmid DNA pBR322 (produced by Takara Shuzo Co., Ltd.). 1 μg of each plasmid was added to 10 μl of a mixture solution [50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, and 1mM dithiothreitol] in a separate tube. Following addition of 2 units each of Hind III and Sal I (which are produced by Takara Shuzo Co., Ltd.), the sample was cleaved at 37 °C for 1 hour and was then extracted with phenol and precipitted with ethanol in accordance with the conventional manner, so that precipitates were obtained. The precipitates were dissolved in 10 μl of a ligation buffer [20 mM $MgCl_2$, 66mM Tris-HCl buffer (pH 7.6), 1mM ATP, and 15 mM dithiothreitol]. After 1 unit of T4 DNA ligase (produced by Takara Shuzo Co., Ltd.) was added, the sample was ligated at 20 °C for 4 hours. Subsequently, the sample solution thus reacted was transformed into E. coli strain JM 101 (ATCC 33876) in accordance with the transformation method (J. Bacteriology, 119: 1,072-1,074 (1974)). Then the E. coli was examined for drug tolerance (ampicillin resistance and tetracycline sensitivity) as well as for β-galactosidase activity, so that a transformant strain was obtained.

Recombinant plasmid DNA contained in the transformant strain was named pMCE10. The E. coli strain JM101 carrying the recombinant plasmid DNA pMCE10 was incubated at 37 C for 16 to 24 hours in a medium consisting of 1 % (W/V) tryptone, 0.5 % (W/V) yeast extract, and 0.5 % (W/V) NaCl. Then, 20 ml culture liquid of the E. coli strain JM 101 (pMCE10) was inoculated into 1 l of a medium of the same composition as described above and was incubated at 37 C for 3 hours under shaking. Subsequently, 0.2 g

chloramphenicol was added and the incubation was continued for further 20 hours at the same temperature, so that a culture liquid was obtained.

Then, the culture liquid was centrifuged at 6,000 r.p.m. for 10 min., to give 2 g wet bacteria bodies. The bacteria were suspended in 20 ml of 350 mM Tris-HCl buffer (pH 8.0) containing 25 % (W/V) sucrose, followed by addition of 10 mg lysozyme, 8 ml of 0.25 M EDTA solution (pH 8. 0), and 8 ml of 20 % (W/V) sodium dodecyl sulfate solution, and the mixture was kept at 60 °C for 30 min., whereby a solution of lysed bacteria was obtained.

13 ml of 5 M NaCl was added to the solution of lysed bacteria, and this mixture was kept at 4 °C for 16 hours and then centrifuged at 15,000 r.p.m. for 30 min. according to the conventional manner, whereby an extract solution was obtained. This extract was subjected to phenol extraction and ethanol precipitation in accordance with the conventional manner, thereby giving precipitates.

Subsequently, the precipitates were dried under reduced pressure as usually carried out, and were then dissolved in 6 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, followed by addition of 6 g cesium chloride and 0.2 ml (10 mg/ml) ethidium bromide solution. Then, the sample mixture was subjected to equilibrium density-gradient centrifugation at 39,000 r.p.m. for 42 hours using a ultracentrifuge in accordance with the conventional manner, whereby a sample composed of the recombinant plasmid DNA pMCE10 us isolated. Then, the sample was extracted with n-butanol to remove ethidium bromide. The DNA solution was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, so that 500 μg of purified recombinant plasmid DNA pMCE10 were obtained.

(6) Preparation of Vector DNA

15 μg of the recombinant plasmid DNA pMCE10 thus prepared were dissolved in 90 μl of TE buffer whose composition is described in (4) above, followed by addition of 10 μl of a Med buffer [100 mM Tris-HCl buffer (pH 7.5), 100 mM $MgCl_2$, 10 mM dithiothreitol, and 500 mM NaCl].
After 30 units of restriction enzyme Acc I (produced by Takara Shuzo Co., Ltd.) were further added, the sample was digested at 37 °C for 1 hour, whereby a cleaved sample was obtained. 100 μl of water-saturated phenol was added thereto for removal of proteins, and then an aqueous layer was recovered. To this aqueous layer were added 1/10 volume of 3 M sodium acetate (pH 7.5) and two volumes of cold ethanol, and the mixture was kept at -70 °C for 15 min. and then centrifuged at 12,000 r.p.m. for 10 min., whereby DNA was recovered.

The DNA was dissolved in 10 μl of TE buffer, followed by addition of 15 μl of a mixture [280 mM sodium cacodylate (pH 6.8), 60 mM Tris-HCl buffer (pH 6.8), and 2 mM cobalt chloride], 5 μl of a (5 mM dGTP-containing) tailing mixture (described in (4) above), and 5 units of terminal transferase (produced by Takara Shuzo Co., Ltd.), and the sample mixture was allowed to react at 37 °C for 15 min. The rest of the procedure was carried out as in the c-DNA tailing reaction described in (4) above, whereby the recombinant plasmid DNA pMCE10 with a deoxyguanosine tail at the Acc I site was prepared.

Separately, the recombinant plasmid DNA pUC19 with a deoxyguanosine tail at the Pst I site was also prepared as follows.

30 μg of plasmid DNA pUC19 (produced by Takara Shuzo Co., Ltd.) was dissolved in 350 μl TE buffer. Following addition of 40 μl of Med buffer and 120 units of restriction enzyme Pst I (produced by Takara Shuzo Co., Ltd.), the sample was incubated at 37 °C for 1 hour. After digestion, the mixture was extracted with phenol. Then, DNA was precipitated with ethanol, whereby DNA was recovered.

The DNA thus obtained was dissolved in 35 μl of TE buffer, followed by addition of 50 μl of a mixture [280 mM sodium cacodylate (pH 6.8), 60 mM Tris-HCl buffer (pH 6. 8), and 1 mM cobalt chloride], 19 μl of a (dGTP-containing) tailing mixture described in (4) above, and 60 units of terminal transferase (produced by Takara Shuzo Co., Ltd.). The sample mixture was allowed to react at 37 °C for 10 min. According to the conventional manner, the sample mixture was treated with phenol and DNA was recovered by ethanol precipitation.

(7) Annealing and Transformation

15 ng of synthesized c-DNA and 200 ng each of the two types of vector DNA prepared in the above method were dissolved in 35 μl of an annealing solution [10 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl, and 1 mM EDTA] in a separate tube, and the mixture was allowed to stand at 65 °C for 2 min., at 46 C for 2 hours, at 37 °C for 1 hour, and then at 20 C for 18 hours, whereby the c-DNA and the vector DNA were annealed. The constructs were transformed into E. coli strain DH1 (ATCC 33849) according to the method as described by Hana-han (DNA Cloning, 1: 109-135 (1985)), and c-DNA libraries were prepared in which

the plasmid DNA pUC19 and the recombinant plasmid DNA pMCE10, respectively, are vectors.

(8) Screening of Luciferase c-DNA

The Acc I site of the recombinant plasmid DNA pMCE10 was located in the region coding for an E. coli β-galactosidase gene, so that the c-DNA having been inserted into this region produced a fusion protein bound to β-galactosidase. The promoter of the β-galactosidase gene of recombinant plasmid DNA pMCE10 had been replaced by that of the tryptophan gene of E. coli as described previously.

96 colonies from the c-DNA library in which the recombinant plasmid DNA pMCE10 is a vector were incubated at 37 °C for 10 hours under shaking in an M9 casamino acid medium (Molecular Cloning, pp. 440-441, Cold Spring Harbor Laboratory (1982)) containing (10 μg/ml) thiamine. Then, the bacteria cells were collected according to the conventional manner and then suspended in 200 μl of sample buffer for SDS-PAGE as described in (2) above, and the suspension was boiled over 5 min. at 100 °C.

40 μl of the suspension was electrophoresed in a 7.5 % (W/V) polyacrylamide gel in accordance with the conventional method. After electrophoresis, the proteins developed in the gel were transferred to a nitrocellulose filter according to the Western blot analysis (Anal. Biochem, 112: 195 (1981)). This nitrocellulose filter was treated with anti-luciferase serum using an immunoblot assay kit (produced by Bio-Rad) according to the method recommended by manufacturer's instructions. That is, a nitrocellulose filter was shaken at 25 °C for 30 min. in 100 ml of 3 % (W/V) blocking solution [in which 3 % (W/V) gelatin is dissolved in a TBS buffer (20 mM Tris-HCl buffer (pH 7.5), 500 mM NaCl]. Then, the nitrocellulose filter was transferred to 25 ml of a primary antibody solution [in which luciferase anti-serum is diluted 1:25 (V/V) with a TBS buffer containing 1 % (W/V) gelatin] and shaken at 25 °C for 90 min. This filter was then transferred to 100 ml of Tween-20 Wash [in which 0.05 % (W/V) Tween-20 is dissolved in TBS buffer] and shaken at 25 °C for 10 min., and this washing procedure was repeated one more time. Subsequently, the nitrocellulose filter thus treated was transferred to 60 ml of a secondary antibody solution [in which a horseradish peroxidase-labelled anti-rabbit antibody (produced by Bio-Rad) is diluted 1:3000 (V/V) with TBS buffer containing 1 % (W/V) gelatin], followed by shaking at 25 °C for 60 min.
Then, the nitrocellulose filter was washed twice with 100 ml of Tween-20 Wash, and this washing was repeated. The nitrocellulose filter thus treated was transferred to 120 ml of a staining solution [i.e. a mixture in which 20 ml of cold methanol having 60 mg of 4-chloro-1-naphthol dissolved is mixed with 100 ml TBS buffer containing 60 μl of 30 % (V/V) hydrogen peroxide], followed by staining at 25 °C for 10 min.

Manipulating 96 colonies as a set, we screened additional three sets of colonies as described above. Two sets were positive. The two sets were further tested: The two sets of colonies were divided into 16 groups (12 colonies per group). The 16 groups were screened as described above. Of these, two groups were positive. 24 colonies were then screened individually as described above. Of these, two positive colonies were found and plasmid DNA of the two colonies was prepared as described in (5) above.

The plasmid DNAs were named pALf2B8 and pALf3A6, respectively.

(9) Screening of Large Luciferase c-DNA (Preparation of a DNA Probe)

100 μg of the recombinant plasmid DNA pALf3A6 were dissolved in 330 μl TE buffer, followed by addition of 40 μl of Low buffer [100 mM Tris-HCl buffer (pH 7:5), 100 mM $MgCl_2$, and 10 mM dithiothreitol], 130 units of Pst I (produced by Takara Shuzo Co., Ltd.), and 120 units of Sac I (produced by Boehringer Mannheim GmbH), and the sample was cleaved at 37 °C for 1.5 hours. The total amount of DNA was separated by electrophoresis in a 0.7 % (W/V) agarose gel. The agarose gel electrophoresis was carried out according to the method described by T. Maniatis et al. (Molecular Cloning, pp. 156-161, Cold Spring Habor Laboratory (1984)). The DNA band containing luciferase c-DNA was cut off and introduced into a dialysis tube. After 2 ml TE buffer was added to the tube, and the tube was sealed. By electroelution, the DNA was eluted from the gel to the buffer. The DNA solution was removed from the tube and extracted with an equal volume of phenol saturated with water. After this sample solution was stirred, the aqueous layer was recovered, and then DNA was recovered by ethanol precipitation in accordance with the conventional manner.

10 μg of the DNA fragment thus obtained was dissolved in 126 μl TE buffer. Following addition of 16 μl Med buffer and 64 units of Sau 3AI (produced by Takara Shuzo Co., Ltd.), the sample was digested at 37 °C for 2 hours, and the total amount of the products were electrophoresed in a 5 % (W/V) polyacrylamide gel, whereby DNA fragments were separated. The polyacrylamide electrophoresis was effected according to the method as described by A. Maxam (Methods in Enzymology, 65: 506 (1980)). 1 μg of the 190 bp Sau 3AI fragment containing the luciferase c-DNA was isolated as described above.

1 µg of the luciferase c-DNA was labeled with [α-^{32}P] dCTP (produced by Amersham) in accordance with the nick translation method. The nick translation was carried out using a kit produced by Takara Shuzo Co., Ltd. in accordance with the method described in J. Mol. Biol., 113: 237-251 (1977) and Molecular Cloning, 109-112, Cold Spring Habor laboratory (1982) as recommended by the manufacturer's instructions.

(10) Screening of large Luciferase c-DNA (Colony Hybridization)

By use, as a probe, of the ^{32}P-labelled luciferase c-DNA fragment as prepared above, the c-DNA library of the Photinus pyralis tails,in which the recombinant plasmid DNA pUC19 is a vector, was screened in accordance with the colony hybridization method (Protein, Nucleic Acid, Enzyme (Japan), 26: 575-579 (1981)), whereby colonies containing luciferase c-DNA were obtained. A recombinant plasmid DNA contained in one of the colonies was named pALf3, and a plasmid DNA was prepared according to the method as described in (5) above. The E. coli carrying said recombinant plasmid DNA was named E. coli DH1 (pALf3). Said transformant E. coli has been deposited as ATCC 67462.

The above recombinant plasmid DNA pALf3 was digested with one or two enzymes from the group consisting of Xba I, Hind III, Bam HI, Eco RI and Pst I (which all are produced by Takara Shuzo Co., Ltd.). The obtained DNA fragments were analyzed for mobility pattern by agarose gel electrophoresis, and the resulting patterns were compared with the standard mobility pattern of DNA fragments prepared by digesting a molecular weight marker, λ-DNA (produced by Takara Shuzo Co., Ltd.) with Hind III. The molecular weight of the luciferase cDNA fragment thus determined was 1,700 bp. The restriction enzyme map of the above plasmid is as set forth in Fig. 1.

(11) Preparation of m-RNA of Luciola cruciata

10 g of alive fireflies, Luciola cruciata (purchased from K.K. Seibu Department Store) were frozen in a supercryogenic temperature refrigerator, and the tails of the fireflies were cut off by means of scissors. 18 ml of a guanidine isocyanate solution was added to 2 g of the tails thus obtained, and 1.1 mg of RNA were prepared according to the method as described in (1) above. 1.1 mg of the RNA were applied to oligo(dT)-cellulose column chromatography in accordance with the method as described in (1) above, whereby 30 µg of m-RNA of the Luciola cruciata tails were prepared.

(12) Construction of a c-DNA Library of Luciola cruciata Tails

Synthesis of c-DNA was carried out according to the method (Mol. Cell Biol., 2: 161 (1982) and Gene, 25: 263 (1983)) as recommended by the manufacturer's (Amersham) instructions. 0.9 µg double-stranded c-DNA was prepared from 2 µg RNA of Luciola cruciata tails. A polydeoxycytidine tail was attached to 0.3 µg of the c-DNA according to the method as described in (4) above.

According to the method as described in (7) above, 20 ng of the c-DNA were annealed to 500 ng of the plasmid DNA pUC19 with a polyguanosine tail attached at the Pst I site as prepared in (6) above. The resulting DNA was transformed into E. coli strain DH1 (ATCC 33849) according to the method as described by Hana-han (DNA Cloning, 1: 109-135(1985)], so that the c-DNA of Luciola cruciata tail was prepared.

(13) Screening of Luciferase c-DNA Derived from Luciola cruciata

10 µg of the transformant plasmid DNA pALf3 obtained in (10) above were dissolved in 90 µl TE buffer. Following addition 25 units of restriction enzyme Eco RI and 25 units of restriction enzyme Cla I (which are produced by Takara Shuzo Co., Ltd.), the DNA was cleaved at 37 °C for 2 hours. The 800 bp Eco RI/Cla I DNA fragment containing luciferase c-DNA derived from Photinus pyralis (American firefly) was isolated from the cleaved recombinant plasmid DNA pALf3 according to the method of agarose gel electrophoresis as described (9) above, whereby 1 µg of Eco RI/Cla I DNA fragment was obtained. 1 µg of the DNA was labeled with [α-^{32}P] dCTP (produced by Amersham) in accordance with the nick translation method as described in (9) above. By use as a probe of ^{32}P-labelled Eco RI/Cla I DNA fragment, E. coli carrying Luciferase c-DNA derived from Luciola cruciata was selected by screening the c-DNA library of the Luciola cruciata tail in accordance with the colony hybridization method as described in (10) above. As a result, several E. coli colonies hybridized with the probe were obtained. Recombinant plasmid DNA contained in one of the colonies was named pGLf 1, and the recombinant plasmid DNA was isolated according to the method as described in (5) above. The E. coli strain carrying said recombinant plasmid DNA was named E. coli DH1 (pGLf 1). Said transformant strain has been deposited as ATCC 67482.

The recombinant plasmid DNA pGLf 1 was digested with one or two enzymes from the group consisting of Hpa I, Hind III, Eco RV, Dra I, Afl II, Hinc II, and Pst I (all of which are produced by Takara Shuzo Co., Ltd.) and Ssp I (produced by New England Bio-Lab). The obtained DNA fragments were analyzed for mobility pattern by agarose gel electrophoresis, and the resulting pattern was compared with the standard mobility pattern of DNA fragments having been prepared by digesting a molecular weight marker, λ-phage DNA (produced by Takara Shuzo Co., Ltd.) with Hind III. As a result, the molecular weight thus determined was 2,000 bp. The restriction enzyme map of the above plasmid is as set forth in Fig. 2.

(14) Analysis of the Base Sequence of the Luciferase c-DNA Derived from Luciola cruciata

10 μg of the recombinant plasmid DNA pGLf 1 were digested with restriction enzyme Pst I (produced by Takara Shuzo Co., Ltd.), whereby 2.5 μg of 2.0 kb DNA fragment containing the luciferase c-DNA were obtained. The DNA fragment was inserted into the Pst I site of plasmid DNA pUC 119 (produced by Takara Shuzo Co., Ltd.), and the resulting plasmid DNAs were named pGLf 2 and pGLf 3 depending on the orientation of the inserted c-DNA. Cleavage with Pst I of recombinant plasmid DNA pGLf 1 and plasmid DNA pUC 119 (the method as described in (6) above), isolation of the luciferase c-DNA fragment by agarose gel electrophoresis (the method as described in (9) above), ligation of plasmid DNA pUC 119 with the luciferase c-DNA fragment (the method as described in (5) above), transformation of E. coli strain JM101 (ATCC 33876) with the construct (the method as described in (5) above), and preparation of recombinant plasmid DNAs pGLf 2 and pGLf 3 (the method as described in (5) above) were carried out as described before.

Then, plasmid DNAs with a variety of deletions introduced into the luciferase c-DNA were prepared from the recombinant plasmid DNAs pGLf 2 and pGLf 3 using a deletion kit for kilosequence (produced by Takara Shuzo Co., Ltd.) according to the method as described by Henikoff [Gene, 28: 351-359 (1984)]). These plasmid DNAs were introduced into E. coli strain JM 101 (ATCC 33876) according to the method as described in (5) above. The E. coli thus obtained was infected with helper phage M13K07 (manufactured by Takara Shuzo Co., Ltd.), to prepare single-stranded DNA according to the method as described by Messing [Methods in Enzymology, 101: 20 to 78 (1983)]. The sequencing by the obtained single-stranded DNA using an M13 sequencing kit (manufactured by Takara Shuzo Co., Ltd.) was carried out in accordance with the Messing method (see above). Gel electrophoresis for the analysis of base sequence was carried out in 8 % (W/V) polyacrylamide gel (manufactured by Fuji Film Co., Ltd.).

The whole base sequence of only the luciferase c-DNA derived from Luciola cruciata and the amino acid sequence deduced from the c-DNA are set forth in Sequence No. 1 and Sequence No. 2, respectively.

(15) Construction of Recombinant Plasmid DNA pGLf 37

First, an explanation is given to the preparation of the DNA fragment comprising vector DNA and the luciferase gene (from which the base sequence encoding 9 amino acids from the N-terminal has been deleted) originating in Luciola cruciata. 1 μg of recombinant plasmid DNA pGLf 1 was dissolved in 90 μl water, followed by addition of 10 μl Med buffer and 20 units of Pst 1 (produced by Takara Shuzo Co., Ltd.), and the mixture was digested at 37 °C for 2 hours. To the sample solution thus digested was added an equal volume of water-saturated phenol, and the sample was precipitated with ethanol in accordance with the conventional manner. According to the method as described in (5) above, the thus obtained precipitates were ligated and transformed into E. coli JM 101 (ATCC 33876).

According to the method as described in (5) above, DNA was isolated from the resulting transformant. The DNA was digested with one or two enzymes from the group consisting of restriction enzymes Ssp I, Eco RV, and Pst I, and the recombinant plasmid which is opposite to the original recombinant plasmid pGLf 1 in respect of the orientation of the c-DNA was selected, and it was named pGLf 10.

10 μg of the recombinant plasmid DNA pGLf 10 were dissolved in 90 μl water. After 10 μl Med buffer and 10 units of Ssp I (produced by New England Bio-Lab) were added to the solution, the mixture was incubated at 37 °C over 30 min., whereby partially digested products were obtained. From the products, there were isolated 2 μg of 4.0 kb DNA fragment comprising most of the vector DNA and the luciferase gene lacking the base sequence encoding 9 amino acids from the N-terminal.

Subsequently, 1 μg of the DNA fragment was dissolved in 95 μl water. Following addition of 5 μl of 1 M Tris-HCl buffer (pH 8.0) and 1 μl (0.3 unit) of alkali phosphalase (produced by Takara Shuzo Co., Ltd.), the mixture was incubated at 65 °C for 1 hour and was then subjected to protein removal treatment and ethanol precipitation in accordance with the conventional manner, and then 1 μl of 4.0 kb DNA fragment dephosphorylated at both ends was obtained.

10 μg of plasmid DNA pKN 206 [which is disclosed in Agric. Biol. Chem., 50: 271-279 (1986)] were dissolved in 90 μl water. After 10 μl Med buffer and 20 units of Cla I (produced by Takara Shuzo Co., Ltd.) were added to the solution, the mixture was incubated at a temperature of 37 °C for 2 hours, so that completely digested sample was obtained. 10 units of Ssp I as stated were added to the sample, followed by incubation at a temperature of 37 °C for 30 min., whereby products partially digested with Ssp I were obtained. Then, the products were subjected to protein removal treatment (by phenol extraction) and ethanol precipitation in accordance with the conventional manner. The precipitates obtained were dissolved in 100 μl TE buffer, and 500 b DNA fragment containing almost all the trp promoter was isolated according to the manner as described in (9) above.

Next, the preparation of the synthetic DNA is explained.

The luciferase gene contained in said 4.0 kb DNA fragment, on the basis of the base sequence, lacked a nucleotide sequence encoding 9 amino acids from the N-terminal.

The trp promoter contained in the above 500 b DNA fragment lacked part of the sequence between the SD and the ATG. In order to fill the sequence encoding 9 amino acids from the N-terminal of luciferase and the sequence between the SD and the ATG of the trp promoter, the following two types of synthetic DNAs (Sequences Nos. 3 and 4) were synthesized using a System 1 Plus DNA synthesizer manufactured by Beckman.

From each of the two types of synthetic DNAs, 20 μg of purified synthetic DNA was prepared using NENSORB PREP manufactured by DuPont. 1 μg each of the purified synthetic DNA was dissolved in 45 μl water, followed by addition of 5 μl of an 10x kination buffer [0.5 M Tris-HCl buffer (pH 7.6), 0.1 M $MgCl_2$, 50 mM dithiothreitol, and 10 mM ATP] and 10 units (1 μl) of T4 polynucleotide kinase (produced by Takara Shuzo Co., Ltd.). Subsequently, the sample was incubated at a temperature of 37 °C for 1 hour and then subjected to protein removal treatment (by phenol extraction) and ethanol precipitation in accordance with the conventional manner, and 1 μg of each of the two types of synthetic DNAs with 5' end phosphorylated was obtained. Then, ligation reaction was made for the preparation of the objective plasmid DNA.

1 μg of the above dephosphorylated 4.0 Kb DNA fragment containing the luciferase gene (from which the base sequence encoding 9 amino acids from the N-terminal has been deleted) and vector DNA, 1 μg of the above 500 b DNA fragment containing the trp promoter, and 0.1 μg of each of the two types of synthetic phosphorylated DNA were dissolved in 8 μl water.

Following addition of 1 μl of an 10x ligation buffer [200 mM $MgCl_2$, 660 mM Tris-HCl buffer (pH 7.6), 10 mM ATP, and 150 mM dithiothreitol] and 1 unit (1 μl) of T4DNA ligase (produced by Takara Shuzo Co., Ltd.), the mixture solution was allowed to react over 16 hours at a temperature of 16 °C According to the method as described in (5) above, the reacted solution was transformed into E. coli JM101 (ATCC 33876) and, according to the method as described in (5) above, plasmid DNA was isolated from the resulting transformant and then digested with one or two enzymes from the group consisting of restriction enzymes Ssp I. Eco RV, and Pst I. The digested products were developed by electrophoresis in a 0.7 % agarose gel, so that the plasmid containing the trp promoter and the luciferase cDNA was obtained, and this recombinant plasmid was named pGLf 37. The E. coli carrying said plasmid was named E. coli JM101 (pGLf 37).

(16) Mutagenesis of Recombinant Plasmid DNA pGLf37

30 μg of the recombinant plasmid DNA pGLf37 were dissolved in 100 μl of a hydroxylamine solution [0.8 M hydroxylamine chloride, 0.1 M phosphate buffer (pH 6.0), and 1 mM EDTA] and then subjected to mutation treatment at 65 °C for 2 hours. After incubation, DNA was precipitated with ethanol in a conventional way, thereby giving precipitates. The precipitates recovered were dissolved in TE buffer [10 mM Tris-HCl buffer (pH 7.5) and 1 mM EDTA] and transformed into E. coli strain JM101 (ATCC 33876) according to the Hana-han method [DNA Cloning, 1: 109-135 (1985)]. Then, said microorganism was inoculated into an LB-amp agar media [1 % (W/V) bactotrypton, 0.5 % (W/V) yeast extract, 0.5 % (W/V) NaCl, (50 μg/ml) ampicillin, and 1.4 % (W/V) agar)], followed by incubation at 37 °C. Colonies came to appear after 12 hours' incubation, and they were incubated for further 18 hours under shaking at 37 °C in 3 ml LB-amp media [1 % (W/V) bactotrypton, 0.5 % (W/V) yeast extract, 0.5 % (W/V) NaCl, and (50 μg/ml) ampicillin]. 0.5 ml of the culture liquid was inoculated into 10 ml of an LB-amp medium of the same composition as described above, followed by incubation at 37 °C for 4 hours under shaking. Subsequently, each of the culture liquids was centrifuged at 8,000 r.p.m. for 10 min., whereby 20 mg of wet bacteria bodies were obtained from each culture liquid.

The recovered bacteria were suspended in 0.9 ml of a buffer consisting of 0.1 M KH_2PO_4 (pH 7.8), 2 mM EDTA, 1 mM dithiothreitol, and 0.2 mg/ml protamine sulfate, followed by addition of 100 μl of 10 mg/ml lysozyme solution, and the suspension was placed on ice for 15 min. Then, this suspension was frozen in

methanol on dry ice, and was then allowed to stand at a temperature of 25 °C, thereby melting completely. This solution was centrifuged at 12,000 r.p.m. for 5 min., whereby 1 ml crude enzyme was obtained as supernatant.

The resulting crude enzyme solution containing luciferase was heated at 50 °C for 10 min., and 10 µl of the crude enzyme was examined for titer in accordance with the method as disclosed in Japanese Patent Appln. LOP Publication No. 141,592/1989. As a result, it was found that the firefly luciferase thus obtained is superior in thermostability to the wild-type luciferase of GENJI firefly.

The crude enzyme solution was further purified according to the method as disclosed in Japanese Patent Appln. LOP Publication No. 141,592/1989 and then heated in the same way as above. The enzyme thus purified was examined for titer, and the result indicated that this enzyme is superior in thermostability to the purified wild-type luciferase. The thus obtained recombinant plasmid DNA into which the gene encoding the thermostable luciferase is inserted was named pGLf37T-M-2. The E. coli strain JM 101 (pGLf37T-M-2) i.e. E. coli transformed with said recombinant plasmid DNA, has been deposited under the accession No. FERM BP-3452 with the Fermentation Research Institute, the Agency of Industrial Science of Technology.

In the mutant firefly luciferase thus obtained, threonine at the 217-position of the amino acid sequence of the wild-type firefly luciferase is replaced by isoleucine. 100 µl of an enzyme solution containing 100 Kcount of the present purified enzyme [10 mM phosphate buffer (pH 7.6) containing 10 % saturated ammonium sulfate, 0.2 mM disodium ethylenediaminetetra acetate, and 0.2 % (W/V) albumin] was examined for residual enzyme activity after kept at a temperature of 50 °C for 60 min. As a result, it was found that the present enzyme keeps 65 % of residual enzyme activity under the above conditions.

As a control, the wild-type firefly luciferase was examined for residual enzyme activity in the same way as described above. However, no activity could be found in this enzyme.

(17) Site-specific Mutagenesis

Next, a method for replacement of threonine, i.e. the amino acid at the 217-position of the amino acid sequence of luciferase of GENJI firefly, by a hydrophobic amino acid valine or leucine is described.

10 µg of recombinant plasmid DNA pGLf 37 were digested with two restriction enzymes Eco RI and Pst I (which are produced from Takara Shuzo Co., Ltd.), whereby 2.5 µg of 2.1 kb DNA fragment containing the luciferase c-DNA were obtained. The DNA fragment was subcloned in plasmid DNA pUC119 (produced by Takara Shuzo Co., Ltd.), and the plasmid DNA thus obtained was named pGPM-1. Then, the recombinant plasmid pGPM-1 was introduced into E. coli JM101 (ATCC 33876), according to the method as described in (5) above.

The E. coli thus obtained was infected with helper phage M13K07 (manufactured by Takara Shuzo Co., Ltd.), and single-stranded DNA was prepared according to the method as described by Messing [Methods in Enzymology, 101: 20-78 (1983)]. Site-specific mutagenesis by the resulting single-stranded DNA was effected using In Vitro Mutagenesis System Version 2.0 (manufactured by Amersham). As primers for site-specific mutagenesis, the following two types of synthetic DNAs were synthesized according to the method as described in (15) above. That is, the synthetic DNA indicated as a primer for valine in Sequence No. 5 and the synthetic DNA indicated as a primer for leucine in Sequence No. 6 were used as primers for site-specific mutagenesis, respectively.

The sequencing of the site-specifically mutagenized genes were carried out using a Dye-Primer Taq Sequencing kit (manufactured by Applied Biosystems, Inc.), and their electrophoresis and analysis was carried out using an AB1373A DNA sequencer (manufactured by Applied Biosystems, Inc.). The site-specifically mutagenated genes thus obtained encode the amino acid sequences in which the amino acid at the 217-position of a wild-type firefly luciferase is replaced by valine or leucine, and the former gene was named pGPM-1-Val and the latter pGMP-1-Leu.

Then, 10 µg each of the site-specifically mutagenated genes pGPM-1-Val DNA and pGPM-1-Leu DNA were digested with two restriction enzymes Eco RI and Pst I (which are produced by Takara Shuzo Co., Ltd.), whereby 2.5 µg each of 2.1 kb DNA fragment containing the luciferase c-DNA were obtained.
Each of the DNA fragment was inserted into luciferase-cDNA-free region of a vector obtained by digestion of recombinant plasmid DNA pGLf37 with two restriction enzymes Eco RI and Pst I (which are produced by Takara Shuzo Co., Ltd.), and the plasmid DNAs thus cloned were named pGLf37-217Val and pGLf37-217Leu, respectively.

Then, the recombinant plasmids pGLf37-217Val and pGLf37-217Leu were introduced into E. coli strain JM101 (ATCC 33876), according to the method as described in (5) above, whereby transformants E. coli JM101 pGLf37-217Val and E. coli JM101 pGLf37-217Leu were obtained. The E. coli JM101 pGLf37-217Val

has been deposited as FERM BP-3647 and the E. coli JM101 pGLf37-217Leu as FERM BP-3648 with the Fermentation Research Institute, Agency of Industry Science and Technology. A crude enzyme solution was obtained from the transformant, according to the method as described in (16) above, and the enzyme was purified according to the method as disclosed in Japanese Patent Appln. LOP Publication No. 141,592/1989. The purified luciferase thus obtained was heated at 50 °C for 60 min., and 10 µl of the enzyme thus treated was measured for residual enzyme activity, according to the method as disclosed in Japanese Patent Appln. LOP Publication No. 141,592/1989. As a result, it was found that the luciferase derived from E. coli JM101 pGLf37-217Val keeps 65 % of residual enzyme activity, and the luciferase derived from E. coli JM101 pGLf37-217Leu keeps 70 % of residual enzyme activity.

(18) Mutagenesis of Recombinant Plasmid DNA pHLf7

Next, a description is provided for a method for the replacement of alanine at the 217-position in the luciferase amino acid sequence of HEIKE firefly (Luciola lateralis) by the hydrophobic amino acid of valine, leucine, or isoleucine.

The recombinant plasmid pHLf7 obtained according to the method as disclosed in Japanese Patent Appln. LOP Publication No. 171,189/1990 was introduced into E. coli JM101 (ATCC 33876), according to the method as described in (5) above. The whole nucleotide sequence of only luciferase c-DNA derived from Luciola lateralis is shown in Sequence No. 7, and the amino acid sequence of the polypeptide deduced from the c-DNA is shown in Sequence No. 8. From the E. coli thus obtained, single-stranded DNA was prepared according to the method as described in (17) above. The site-specific mutagenesis by the resultant single-stranded DNA was effected using In Vitro Mutagenesis System Version-2.0 (manufactured by Amersham).

According to the method as described in (15) above, the following 3 types of synthetic DNAs were synthesized as primers for site-specific mutagenesis. That is, the synthetic DNA indicated as a primer for valine in Sequence No. 9, the synthetic DNA indicated as a primer for leucine in Sequence No. 10, and the synthetic DNA indicated as a primer for isoleucine in Sequence No. 11 were used as primers for site-specific mutagenesis, respectively.

The sequencing of the site-specifically mutagenated gene was carried out using a Dye-Primer Taq Sequencing kit (manufactured by Applied Biosystems, Inc.), and analytical electrophoresis was conducted using an ABI 373A DNA sequencer (manufactured by Applied Biosystems, Inc.). The thus obtained site-specifically mutagenated luciferase genes in the recombinant plasmids encode, in the corresponding gene region, that amino acid at the 217-position of wild-type HEIKE firefly luciferase which has been converted into valine, leucine, and isoleucine, and the recombinant plasmids with such genes were named pHLf7-217Val, pHLf7-217Leu, and pHLf7-217Ile. respectively.

Subsequently, the recombinant plasmids pHLf7-217Val, pHLf7-217Leu, and pHLf7-217Ile, respectively, were transformed into E. coli strain JM101 (ATCC 33876), whereby E. coli JM101 pHLf7-217Val, E. coli JM101 pHLf7-217Leu, and E. coli JM101 pHLf7-217Ile were obtained. The transformants E. coli JM101 pHLf7-217Val, E. coli JM101 pHLf7-217Leu, and E. coli JM101 pHLf7-217Ile were deposited under Accession Nos. FERM BP-3839, FERM BP-3841, and FERM BP-3840, respectively, with the Fermentation Research Institute, the Agency of Industrial Science of Technology. According to the method as described in (16) above, a crude enzyme solution was obtained from each transformant, and it was further purified according to the method as disclosed in Japanese Patent Appln. LOP Publication No. 262,791/1989. The purified luciferase thus obtained was heated at 50 °C for 60 min., and 10 µl of the solution was measured for residual enzyme activity in accordance with the method as disclosed in Japanese Patent Appln. LOP Publication No. 262,791/1989. As a result, it was found that 65 % or more of residual enzyme is kept in every case.

As is evident from the above illustration, the luciferase of the invention was found to be extremely superior in thermostability to the control luciferase.

Effect of the Invention

The present invention provides a gene of a thermostable luciferase of a firefly, recombinant DNA containing said gene, and a process for the preparation of a thermostable luciferase of firefly by a microorganism carrying said recombinant DNA, as well as a novel thermostable luciferase of a firefly thus obtained. According to the present method, a thermostable luciferase of a firefly can be efficiently produced, so that the present invention is extremely useful in industry.

[Sequence Tables]

1. Sequence No. 1

(1) Length of Sequence: 1644

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: cDNA to mRNA

(6) Origin: Luciola cruciata

(7) Sequence:

ATG GAA AAC ATG GAA AAC GAT GAA AAT ATT GTA GTT GGA CCT AAA 45

CCG TTT TAC CCT ATC GAA GAG GGA TCT GCT GGA ACA CAA TTA CGC 90

AAA TAC ATG GAG CGA TAT GCA AAA CTT GGC GCA ATT GCT TTT ACA 135

AAT GCA GTT ACT GGT GTT GAT TAT TCT TAC GCC GAA TAC TTG GAG 180

AAA TCA TGT TGT CTA GGA AAA GCT TTG CAA AAT TAT GGT TTG GTT 225

GTT GAT GGC AGA ATT GCG TTA TGC AGT GAA AAC TGT GAA GAA TTT 270

TTT ATT CCT GTA ATA GCC GGA CTG TTT ATA GGT GTA GGT GTT GCA 315

CCC ACT AAT GAG ATT TAC ACT TTA CGT GAA CTG GTT CAC AGT TTA 360

GGT ATC TCT AAA CCA ACA ATT GTA TTT AGT TCT AAA AAA GGC TTA 405

GAT AAA GTT ATA ACA GTA CAG AAA ACA GTA ACT ACT ATT AAA ACC 450

ATT GTT ATA CTA GAT AGC AAA GTT GAT TAT CGA GGA TAT CAA TGT 495

CTG GAC ACC TTT ATA AAA AGA AAC ACT CCA CCA GGT TTT CAA GCA 540

TCC AGT TTC AAA ACT GTG GAA GTT GAC CGT AAA GAA CAA GTT GCT 585

CTT ATA ATG AAC TCT TCG GGT TCT ACC GGT TTG CCA AAA GGC GTA 630

CAA CTT ACT CAC GAA AAT ACA GTC ACT AGA TTT TCT CAT GCT AGA 675

GAT CCG ATT TAT GGT AAC CAA GTT TCA CCA GGC ACC GCT GTT TTA 720

ACT GTC GTT CCA TTC CAT CAT GGT TTT GGT ATG TTC ACT ACT CTA 765

GGG TAT TTA ATT TGT GGT TTT CGT GTT GTA ATG TTA ACA AAA TTC 810

GAT GAA GAA ACA TTT TTA AAA ACT CTA CAA GAT TAT AAA TGT ACA 855

AGT GTT ATT CTT GTA CCG ACC TTG TTT GCA ATT CTC AAC AAA AGT 900

GAA TTA CTC AAT AAA TAC GAT TTG TCA AAT TTA GTT GAG ATT GCA 945
TCT GGC GGA GCA CCT TTA TCA AAA GAA GTT GGT GAA GCT GTT GCT 990
AGA CGC TTT AAT CTT CCC GGT GTT CGT CAA GGT TAT GGT TTA ACA 1035
GAA ACA ACA TCT GCC ATT ATT ATT ACA CCA GAA GGA GAC GAT AAA 1080
CCA GGA GCT TCT GGA AAA GTC GTG CCG TTG TTT AAA GCA AAA GTT 1125
ATT GAT CTT GAT ACC AAA AAA TCT TTA GGT CCT AAC AGA CGT GGA 1170
GAA GTT TGT GTT AAA GGA CCT ATG CTT ATG AAA GGT TAT GTA AAT 1215
AAT CCA GAA GCA ACA AAA GAA CTT ATT GAC GAA GAA GGT TGG CTG 1260
CAC ACC GGA GAT ATT GGA TAT TAT GAT GAA GAA AAA CAT TTC TTT 1305
ATT GTC GAT CGT TTG AAG TCT TTA ATC AAA TAC AAA GGA TAC CAA 1350
GTA CCA CCT GCC GAA TTA GAA TCC GTT CTT TTG CAA CAT CCA TCT 1395
ATC TTT GAT GCT GGT GTT GCC GGC GTT CCT GAT CCT GTA GCT GGC 1440
GAG CTT CCA GGA GCC GTT GTT GTA CTG GAA AGC GGA AAA AAT ATG 1485
ACC GAA AAA GAA GTA ATG GAT TAT GTT GCA AGT CAA GTT TCA AAT 1530
GCA AAA CGT TTA CGT GGT GGT GTT CGT TTT GTG GAT GAA GTA CCT 1575
AAA GGT CTT ACT GGA AAA ATT GAC GGC AGA GCA ATT AGA GAA ATC 1620
CTT AAG AAA CCA GTT GCT AAG ATG 1644

2. Sequence No. 2

(1) Length of Sequence: 548

(2) Sequence Type: Amino Acid

(3) Topology: Straight-Chain

(4) Sequence Kind: Peptide

(5) Origin of Sequence: Luciola cruciata

(6) Sequence:

Met Glu Asn Met Glu Asn Asp Glu Asn Ile
10

Val Val Gly Pro Lys Pro Phe Tyr Pro Ile
20

30

Glu Glu Gly Ser Ala Gly Thr Gln Leu Arg

40

Lys Tyr Met Glu Arg Tyr Ala Lys Leu Gly

50

Ala Ile Ala Phe Thr Asn Ala Val Thr Gly

60

Val Asp Tyr Ser Tyr Ala Glu Tyr Leu Glu

70

Lys Ser Cys Cys Leu Gly Lys Ala Leu Gln

80

Asn Tyr Gly Leu Val Val Asp Gly Arg Ile

90

Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe

100

Phe Ile Pro Val Ile Ala Gly Leu Phe Ile

110

Gly Val Gly Val Ala Pro Thr Asn Glu Ile

120

Tyr Thr Leu Arg Glu Leu Val His Ser Leu

130

Gly Ile Ser Lys Pro Thr Ile Val Phe Ser

140

Ser Lys Lys Gly Leu Asp Lys Val Ile Thr

150

Val Gln Lys Thr Val Thr Thr Ile Lys Thr

160

Ile Val Ile Leu Asp Ser Lys Val Asp Tyr

170

Arg Gly Tyr Gln Cys Leu Asp Thr Phe Ile

180

Lys Arg Asn Thr Pro Pro Gly Phe Gln Ala

190

Ser Ser Phe Lys Thr Val Glu Val Asp Arg

200

Lys Glu Gln Val Ala Leu Ile Met Asn Ser

210

Ser Gly Ser Thr Gly Leu Pro Lys Gly Val

220

Gln Leu Thr His Glu Asn Thr Val Thr Arg

230

Phe Ser His Ala Arg Asp Pro Ile Tyr Gly

240

Asn Gln Val Ser Pro Gly Thr Ala Val Leu

250

Thr Val Val Pro Phe His His Gly Phe Gly

260

Met Phe Thr Thr Leu Gly Tyr Leu Ile Cys

270

Gly Phe Arg Val Val Met Leu Thr Lys Phe

280

Asp Glu Glu Thr Phe Leu Lys Thr Leu Gln

290

Asp Tyr Lys Cys Thr Ser Val Ile Leu Val

300

Pro Thr Leu Phe Ala Ile Leu Asn Lys Ser

310

Glu Leu Leu Asn Lys Tyr Asp Leu Ser Asn

330

Leu Val Glu Ile Ala Ser Gly Gly Ala Pro

340

Leu Ser Lys Glu Val Gly Glu Ala Val Ala

350

Arg Arg Phe Asn Leu Pro Gly Val Arg Gln

360

Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala

370

Ile Ile Ile Thr Pro Glu Gly Asp Asp Lys

380

Pro Gly Ala Ser Gly Lys Val Val Pro Leu

390

Phe Lys Ala Lys Val Ile Asp Leu Asp Thr

400

Lys Lys Ser Leu Gly Pro Asn Arg Arg Gly

410

Glu Val Cys Val Lys Gly Pro Met Leu Met

420

Lys Gly Tyr Val Asn Asn Pro Glu Ala Thr

430

Lys Glu Leu Ile Asp Glu Glu Gly Trp Leu

440

His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu

450

Glu Lys His Phe Phe Ile Val Asp Arg Leu

460

Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln

Val Pro Pro Ala Glu Leu Glu Ser Val Leu

470

Leu Gln His Pro Ser Ile Phe Asp Ala Gly

480

Val Ala Gly Val Pro Asp Pro Val Ala Gly

490

Glu Leu Pro Gly Ala Val Val Val Leu Glu

500

Ser Gly Lys Asn Met Thr Glu Lys Glu Val

510

Met Asp Tyr Val Ala Ser Gln Val Ser Asn

520

Ala Lys Arg Leu Arg Gly Gly Val Arg Phe

530

Val Asp Glu Val Pro Lys Gly Leu Thr Gly

540

Lys Ile Asp Gly Arg Ala Ile Arg Glu Ile

Leu Lys Lys Pro Val Ala Lys Met

3. Sequence No. 3

(1) Length of Sequence: 32

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: Other Nucleic Acid. Synthetic DNA Primer

(6) Sequence:

CGA CAA TGG AAA ACA TGG AAA ACG ATG AAA AT

4. Sequence No. 4

(1) Length of Sequence: 30

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: Other Nucleic Acid, Synthetic DNA Primer

(6) Sequence:

ATT TTC ATC GTT TTC CAT GTT TTC CAT TGT

5. Sequence No. 5

(1) Length of Sequence: 38

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: Other Nucleic Acid, Synthetic DNA Primer

(6) Sequence:

CTC TAG CAT GCG AAA ATC TAG TGA CTA CAT TTT CGT GA

6. Sequence No. 6

(1) Length of Sequence: 38

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: Other Nucleic Acid, Synthetic DNA Primer

(6) Sequence:

CTC TAG CAT GCG AAA ATC TAG TGA CGA CAT TTT CGT GA

7. Sequence No. 7.

(1) Length of Sequence: 1644

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: cDNA to mRNA

(6) Origin: Luciola lateralis

(7) Sequence:

30
ATG GAA AAC ATG GAG AAC GAT GAA AAT ATT

60
GTG TAT GGT CCT GAA CCA TTT TAC CCT ATT

90
GAA GAG GGA TCT GCT GGA GCA CAA TTG CGC

120
AAG TAT ATG GAT CGA TAT GCA AAA CTT GGA

150
GCA ATT GCT TTT ACT AAC GCA CTT ACC GGT

180
GTC GAT TAT ACG TAC GCC GAA TAC TTA GAA

210
AAA TCA TGC TGT CTA GGA GAG GCT TTA AAG

240
AAT TAT GGT TTG GTT GTT GAT GGA AGA ATT

270
GCG TTA TGC AGT GAA AAC TGT GAA GAA TTC

300
TTT ATT CCT GTA TTA GCC GGT TTA TTT ATA

330
GGT GTC GGT GTG GCT CCA ACT AAT GAG ATT

360

TAC ACT CTA CGT GAA TTG GTT CAC AGT TTA

390

GGC ATC TCT AAG CCA ACA ATT GTA TTT AGT

420

TCT AAA AAA GGA TTA GAT AAA GTT ATA ACT

450

GTA CAA AAA ACG GTA ACT GCT ATT AAA ACC

480

ATT GTT ATA TTG GAC AGC AAA GTG GAT TAT

510

AGA GGT TAT CAA TCC ATG GAC AAC TTT ATT

540

AAA AAA AAC ACT CCA CAA GGT TTC AAA GGA

570

TCA AGT TTT AAA ACT GTA GAA GTT AAC CGC

600

AAA GAA CAA GTT GCT CTT ATA ATG AAC TCT

630

TCG GGT TCA ACC GGT TTG CCA AAA GGT GTG

660

CAA CTT ACT CAT GAA AAT GCA GTC ACT AGA

690

TTT TCT CAC GCT AGA GAT CCA ATT TAT GGA

720

AAC CAA GTT TCA CCA GGC ACG GCT ATT TTA

750

ACT GTA GTA CCA TTC CAT CAT GGT TTT GGT

780

ATG TTT ACT ACT TTA GGC TAT CTA ACT TGT

810

GGT TTT CGT ATT GTC ATG TTA ACG AAA TTT

840

GAC GAA GAG ACT TTT TTA AAA ACA CTG CAA

870

GAT TAC AAA TGT TCA AGC GTT ATT CTT GTA

900

CCG ACT TTG TTT GCA ATT CTT AAT AGA AGT

930

GAA TTA CTC GAT AAA TAT GAT TTA TCA AAT

960

TTA GTT GAA ATT GCA TCT GGC GGA GCA CCT

990

TTA TCT AAA GAA ATT GGT GAA GCT GTT GCT

1020

AGA CGT TTT AAT TTA CCG GGT GTT CGT CAA

1050

GGC TAT GGT TTA ACA GAA ACA ACC TCT GCA

1080

ATT ATT ATC ACA CCG GAA GGC GAT GAT AAA

1110

CCA GGT GCT TCT GGC AAA GTT GTG CCA TTA

1140

TTT AAA GCA AAA GTT ATC GAT CTT GAT ACT

1170

AAA AAA ACT TTG GGC CCG AAC AGA CGT GGA

1200

GAA GTT TGT GTA AAG GGT CCT ATG CTT ATG

1230

AAA GGT TAT GTA GAT AAT CCA GAA GCA ACA

1260

AGA GAA ATC ATA GAT GAA GAA GGT TGG TTG

1290

CAC ACA GGA GAT ATT GGG TAT TAC GAT GAA

1320

GAA AAA CAT TTC TTT ATC GTG GAT CGT TTG

1350

AAG TCT TTA ATC AAA TAC AAA GGA TAT CAA

1380

GTA CCA CCT GCT GAA TTA GAA TCT GTT CTT

1410

TTG CAA CAT CCA AAT ATT TTT GAT GCC GGC

1440

GTT GCT GGC GTT CCA GAT CCT ATA GCT GGT

1470

GAG CTT CCG GGA GCT GTT GTT GTA CTT GAA

1500

AAA GGA AAA TCT ATG ACT GAA AAA GAA GTA

1530

ATG GAT TAC GTT GCT AGT CAA GTT TCA AAT

1560

GCA AAA CGT TTG CGT GGT GGT GTC CGT TTT

1590

GTG GAC GAA GTA CCT AAA GGT CTC ACT GGT

1620

AAA ATT GAC GGT AAA GCA ATT AGA GAA ATA

CTG AAG AAA CCA GTT GCT AAG ATG

8. Sequence No. 8

(1) Length of Sequence: 548

(2) Sequence Type: Amino Acid

(3) Topology: Straight-Chain

(4) Sequence Kind: Peptide

(5) Origin of Sequence: Luciola lateralis

(6) Sequence:

```
                                                    10
Met Glu Asn Met Glu Asn Asp Glu Asn Ile
                                                    20
Val Tyr Gly Pro Glu Pro Phe Tyr Pro Ile
                                                    30
Glu Glu Gly Ser Ala Gly Ala Gln Leu Arg
                                                    40
Lys Tyr Met Asp Arg Tyr Ala Lys Leu Gly
                                                    50
Ala Ile Ala Phe Thr Asn Ala Leu Thr Gly
                                                    60
Val Asp Tyr Thr Tyr Ala Glu Tyr Leu Glu
                                                    70
Lys Ser Cys Cys Leu Gly Glu Ala Leu Lys
                                                    80
Asn Tyr Gly Leu Val Val Asp Gly Arg Ile
                                                    90
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe
                                                   100
Phe Ile Pro Val Leu Ala Gly Leu Phe Ile
                                                   110
```

Gly Val Gly Val Ala Pro Thr Asn Glu Ile

120

Tyr Thr Leu Arg Glu Leu Val His Ser Leu

130

Gly Ile Ser Lys Pro Thr Ile Val Phe Ser

140

Ser Lys Lys Gly Leu Asp Lys Val Ile Thr

150

Val Gln Lys Thr Val Thr Ala Ile Lys Thr

160

Ile Val Ile Leu Asp Ser Lys Val Asp Tyr

170

Arg Gly Tyr Gln Ser Met Asp Asn Phe Ile

180

Lys Lys Asn Thr Pro Gln Gly Phe Lys Gly

190

Ser Ser Phe Lys Thr Val Glu Val Asn Arg

200

Lys Glu Gln Val Ala Leu Ile Met Asn Ser

210

Ser Gly Ser Thr Gly Leu Pro Lys Gly Val

220

Gln Leu Thr His Glu Asn Ala Val Thr Arg

230

Phe Ser His Ala Arg Asp Pro Ile Tyr Gly

240

Asn Gln Val Ser Pro Gly Thr Ala Ile Leu

250

Thr Val Val Pro Phe His His Gly Phe Gly

260
Met Phe Thr Thr Leu Gly Tyr Leu Thr Cys
270
Gly Phe Arg Ile Val Met Leu Thr Lys Phe
280
Asp Glu Glu Thr Phe Leu Lys Thr Leu Gln
290
Asp Tyr Lys Cys Ser Ser Val Ile Leu Val
300
Pro Thr Leu Phe Ala Ile Leu Asn Arg Ser
310
Glu Leu Leu Asp Lys Tyr Asp Leu Ser Asn
320
Leu Val Glu Ile Ala Ser Gly Gly Ala Pro
330
Leu Ser Lys Glu Ile Gly Glu Ala Val Ala
340
Arg Arg Phe Asn Leu Pro Gly Val Arg Gln
350
Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala
360
Ile Ile Ile Thr Pro Glu Gly Asp Asp Lys
370
Pro Gly Ala Ser Gly Lys Val Val Pro Leu
380
Phe Lys Ala Lys Val Ile Asp Leu Asp Thr
390
Lys Lys Thr Leu Gly Pro Asn Arg Arg Gly
400

Glu Val Cys Val Lys Gly Pro Met Leu Met

410

Lys Gly Tyr Val Asp Asn Pro Glu Ala Thr

420

Arg Glu Ile Ile Asp Glu Glu Gly Trp Leu

430

His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu

440

Glu Lys His Phe Phe Ile Val Asp Arg Leu

450

Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln

460

Val Pro Pro Ala Glu Leu Glu Ser Val Leu

470

Leu Gln His Pro Asn Ile Phe Asp Ala Gly

480

Val Ala Gly Val Pro Asp Pro Ile Ala Gly

490

Glu Leu Pro Gly Ala Val Val Val Leu Glu

500

Lys Gly Lys Ser Met Thr Glu Lys Glu Val

510

Met Asp Tyr Val Ala Ser Gln Val Ser Asn

520

Ala Lys Arg Leu Arg Gly Gly Val Arg Phe

530

Val Asp Glu Val Pro Lys Gly Leu Thr Gly

540

Lys Ile Asp Gly Lys Ala Ile Arg Glu Ile

Leu Lys Lys Pro Val Ala Lys Met

9. Sequence No. 9

(1) Length of Sequence: 36

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: Other Nucleic Acid, Synthetic DNA Primer

(6) Sequence:

AGC GTG AGA AAA ACG CGT GAC GAC ATT TTC ACG AGT

10. Sequence No. 10

(1) Length of Sequence: 36

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: Other Nucleic Acid, Synthetic DNA Primer

(6) Sequence:

AGC GTG AGA AAA ACG CGT GAC CAA ATT TTC ACG AGT

11. Sequence No. 11

(1) Length of Sequence: 36

(2) Sequence Type: Nucleic Acid

(3) Number of Chains: Single-Stranded Chain

(4) Topology: Straight-Chain

(5) Sequence Kind: Other Nucleic Acid, Synthetic DNA Primer

(6) Sequence:

AGC GTG AGA AAA ACG CGT GAC GAT ATT TTC ACG AGT

Claims

1. A DNA sequence of a thermostable luciferase of a firefly, which encodes the amino acid sequence of a wild-type firefly luciferase in which an amino acid at the 217-position or an amino acid at the position equivalent to the 217-position of the luciferase of Luciola cruciata (GENJI firefly) or Luciola lateralis (HEIKE firefly) is replaced by a hydrophobic amino acid.

2. The DNA sequence of a thermostable luciferase of a firefly according to claim 1, wherein the wild-type firefly luciferase is the luciferase of Luciola lateralis (HEIKE firefly) or Luciola cruciata (GENJI firefly).

3. The DNA sequence of a thermostable luciferase of a firefly according to claim 1 or 2, wherein the hydrophobic amino acid is isoleucine, leucine, or valine.

4. A recombinant DNA molecule containing a DNA sequence according to any one of claims 1 to 3.

5. The recombinant DNA molecule according to claim 4, wherein said DNA sequence is under the control of a promoter allowing its expression in a desired host cell.

6. The recombinant DNA molecule according to claim 5, wherein the promoter is the trp promoter.

7. A host cell containing a recombinant DNA molecule according to any one of claims 4 to 6.

8. The host cell according to claim 7 which is a bacterial cell, a yeast cell, an insect cell, a plant cell or a mammalian cell.

9. A process for the preparation of a thermostable luciferase of a firefly, which comprises incubation in a culture medium of a microorganism belonging to the genus Escherichia, carrying the recombinant DNA molecule according to any one of claims 4 to 6 and being capable of producing a thermostable luciferase of a firefly, and subsequent collection of the thermostable luciferase of the firefly from said culture medium.

10. A thermostable luciferase of a firefly, wherein an amino acid at the 217-position of the amino acid sequence of a wild-type firefly luciferase or an amino acid at the position equivalent to the 217-position of the luciferase of Luciola cruciata (GENJI firefly) or Luciola lateralis (HEIKE firefly) is replaced by a hydrophobic amino acid.

11. The thermostable luciferase of a firefly according to claim 10, wherein the wild-type firefly luciferase is the luciferase of Luciola lateralis (HEIKE firefly) or Luciola cruciata (GENJI firefly).

12. The thermostable luciferase of a firefly according to claim 10 or 11, wherein the hydrophobic amino acid is isoleucin, leucin or valine.

FIG.1

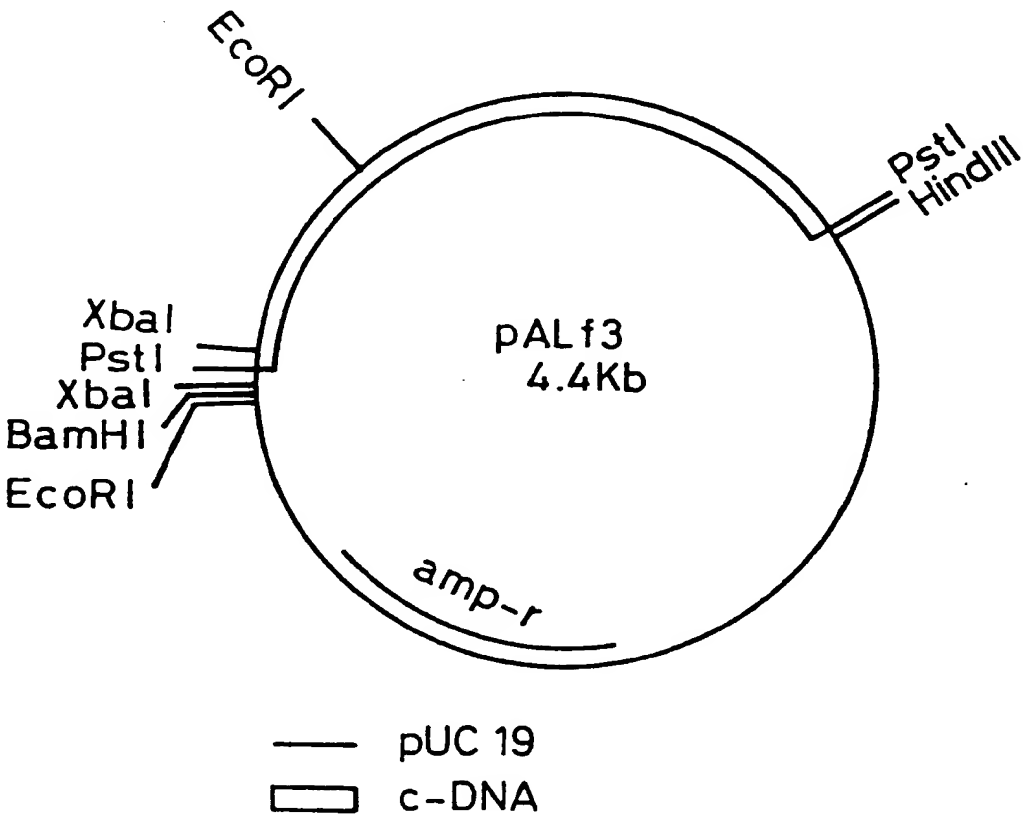
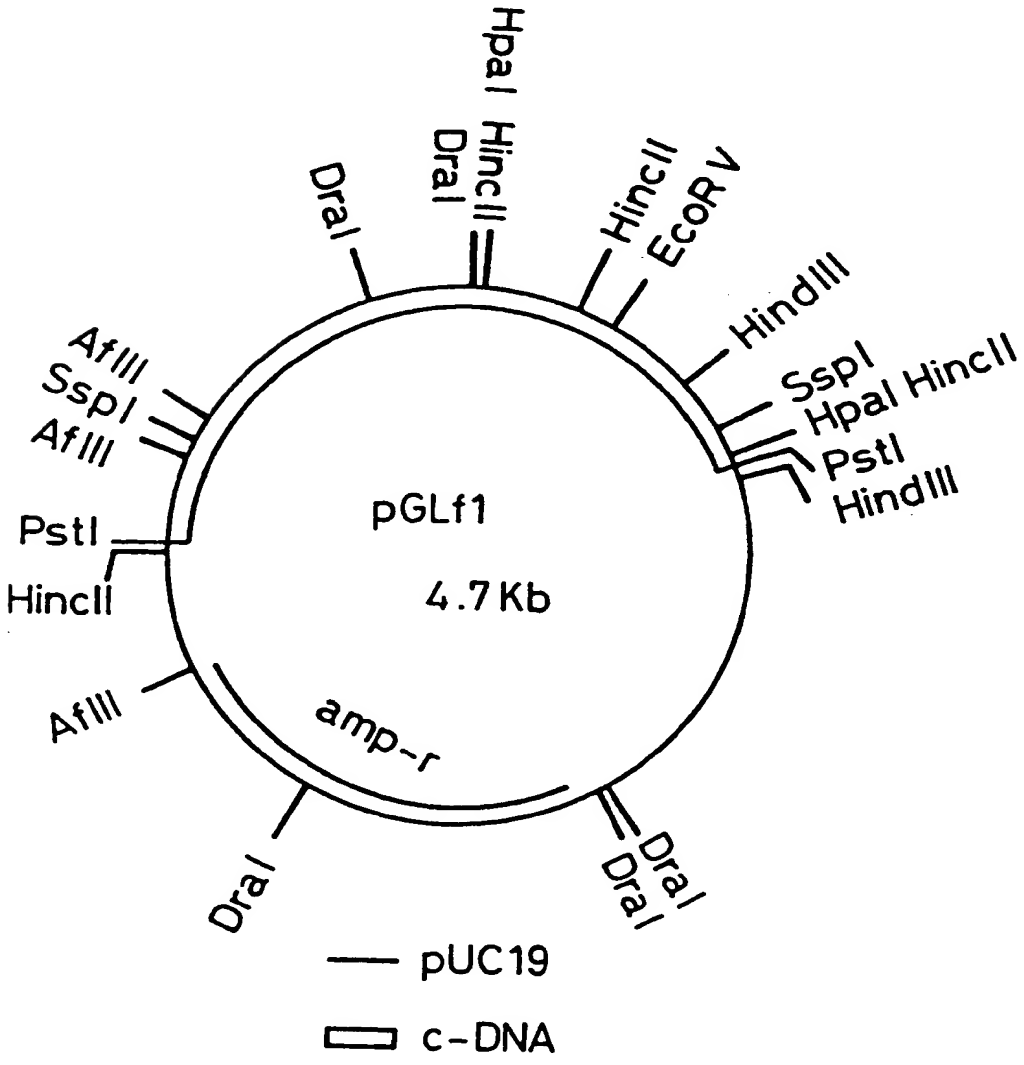

EcoRI
PstI
HindIII
XbaI
PstI
XbaI
BamHI
EcoRI
pALf3
4.4Kb
amp-r
pUC 19
c-DNA

FIG. 2


HpaI
HincII
DraI
DraI
HincII
EcoRV
HindIII
SspI
HpaI HincII
PstI
HindIII
pGLf1
4.7Kb
amp-r
DraI
DraI
DraI
AflII
HincII
PstI
AflII
SspI
AflII
pUC19
c-DNA

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 11 0808

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 318 915 (KIKKOMAN CORP) * the whole document * & JP-A-1 141 592 (...)	1,2,10, 11	C12N15/53 C12N9/02
D			
A	EP-A-0 337 349 (KIKKOMAN CORP) * the whole document * & JP-A-1 262 791 (...)	1,2,10, 11	
D			
A	EP-A-0 275 202 (CHIRON CORP) * abstract; claims 1-20 *	1,4,7,9, 10	
A	CHEMICAL ABSTRACTS, vol. 117, no. 9 Columbus, Ohio, US; abstract no. 85713s, ESCHER, A.; O'KANE, D. J.; SZALAY, A. A. 'Engineering of a bacterial luciferase alpha-beta fusion protein with enhanced activity at 37.degree.C in Escherichia coli' * abstract * & Biolumin. Chemilumin. Proc. Int. Symp., 6th, Meeting Date 1990, 15-18. Edited by: Stanley, Philip E.; Kricka, Larry J. Wiley: Chichester, UK.	1,10	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	16 OCTOBER 1992	GURDJIAN D.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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